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Biomarkers

Evaluation of Circulating Endothelial Cells as Direct Marker of Endothelial Damage in Allo-Transplant Recipients at High Risk of Hepatic Veno-Occlusive Disease/Sinusoidal Obstruction Syndrome

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A B S T R A C T

Sinusoidal obstruction syndrome (SOS), also known as veno-occlusive disease (VOD), is a rare but potentially fatal complication following allogenic hematopoietic cell

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transplantation (allo-HCT). Timely identification of SOS/VOD to allow for prompt treatment is critical, but identifying a VOD-predictive biomarker remains challenging. Given the pivotal role of endothelial dysfunction in SOS/VOD pathophysiology, the CECinVOD study prospectively evaluated levels of circulating endothelial cells (CECs) in patients undergoing allo-HCT with a myeloablative conditioning (MAC) regimen to investigate the potential of CEC level in predicting and diagnosing SOS/VOD. A total of 150 patients from 11 Italian bone marrow transplantation units were enrolled. All participants were age >18 years and received a MAC regimen, putting them at elevated risk of developing SOS/VOD. Overall, 6 cases of SOS/VOD (4%) were recorded. CECs were detected using the Food and Drug Administration-approved CellSearch system, an immunomagnetic selection-based platform incorporating ferrofluid nanoparticles and fluorescent-labeled antibodies, and were defined as CD146+, CD105+, DAPI+, or CD45-. Blood samples were collected at the following time points: before (T0) and at the end of conditioning treatment (T1), at neutrophil engraftment (T2), and at 7 to 10 days postengraftment (T3). For patients who developed VOD, additional samples were collected at any suspected or proven VOD onset (T4) and weekly during defibrotide treatment (T5 to T8). A baseline CEC count >17/mL was associated with an elevated risk of SOS/VOD ($P = .04$), along with bilirubin level >1.5 mg/mL and a haploidentical donor hematopoietic stem cell source. Postconditioning regimen (T1) CEC levels were elevated ($P = .02$), and levels were further increased at engraftment ($P < .0001$). Additionally, patients developing SOS/VOD after engraftment, especially those with late-onset SOS/VOD, showed a markedly higher relative increase (>150%) in CEC count. Multivariate analysis supported these findings, along with a high Endothelial Activation and Stress Index (EASIX) score at engraftment (T2). Finally, CEC kinetics corresponded with defibrotide treatment. After the start of therapy (T4), CEC levels showed an initial increase in the first week (T5), followed by a progressive decrease during VOD treatment (T6 and T7) and a return to pre-SOS/VOD onset levels at resolution of the complication. This prospective multicenter study reveals a low incidence of SOS/VOD in high-risk patients compared to historical data, in line with recent reports. The results from the CECinVOD study collectively confirm the endothelial injury in allo-HCT and its role in in the development of SOS/VOD, suggesting that CEC level can be a valuable biomarker for diagnosing SOS/VOD and identifying patients at greater risk of this complication, especially late-onset SOS/VOD. Furthermore, CEC kinetics may support treatment strategies by providing insight into the optimal timing for discontinuing defibrotide treatment.

INTRODUCTION

Sinusoidal obstruction syndrome (SOS), also known as veno-occlusive disease (VOD), is a severe and potentially life-threatening complication occurring after chemotherapy, conjugated antibodies therapy, and allogenic hematopoietic cell transplantation (allo-HCT) [1,2]. Rarely, it occurs after high-dose radiotherapy [3], liver transplantation [4], or ingestion of toxic alkaloids (toxic injury) [5]. SOS/VOD is marked by painful hepatomegaly, fluid retention with ascites, weight gain, and jaundice [2]. Severe forms of SOS/VOD can swiftly progress to multiorgan dysfunction and are associated with very high mortality (>80%). Early diagnosis and prompt treatment are imperative, although hindered by the challenge of clear diagnostic criteria. Symptoms often overlap with graft-versus-host disease (GVHD), sepsis, and other causes of multiorgan failure. Recently revised diagnostic criteria from the European Society for Blood and Marrow Transplantation (EBMT) [2] aimed to address the limitations of previous criteria, focusing on the early stages of the disease by introducing the concepts of probable, clinical, and proven SOS/VOD. The reported incidence of SOS/VOD varies significantly, ranging between 5% and 15%, depending on the presence of such risk factors as conditioning regimen intensity and the specific diagnostic criteria applied [2,6-9].

After allo-HCT, numerous factors can lead to endothelial activation and dysfunction, including alloreactivity, infections, immunosuppressive agents, engraftment, and proinflammatory cytokines [10–12]. Pathogenetically, SOS/VOD originates from endothelial cell injury and hepatic venule dysfunction owing to the toxic effects of drugs included in conditioning regimens, particularly alkylating agents [13,14]. The activation and

disruption of sinusoidal endothelial cells lead to endothelial detachment and sinusoidal obstruction, resulting in postsinusoidal portal hypertension [15]. Given the central role of endothelial dysfunction in SOS/VOD pathophysiology, various markers of endothelial dysfunction have been investigated in recent years, including L-ficolin, hyaluronic acid, vascular cell adhesion molecule 1, and angiopoietin-2 [16–18]. These biomarkers are considered indirect indicators of endothelial activation or damage and can originate not only from the endothelium itself, but also from other sources, such as leukocytes and platelets [19,20]. Moreover, these biomarkers may be influenced by several conditions, such as renal or liver dysfunction and inflammation [19,21], and require further validation and integration into clinical practice [2].

More recently, the Endothelial Activation and Stress Index (EASIX) has been related to SOS/VOD in 2 independent cohorts [22]. However, EASIX must be considered an indirect marker of endothelium activation or injury and remains to be validated.

The level of circulating endothelial cells (CECs), mature endothelial cells that are shed from vessel walls as a result of pathophysiologic conditions affecting endothelial integrity [23–25], has been identified as a reliable biomarker of endothelial damage [26] and acute GVHD [27] in allo-HCT recipients. Given the promise of CECs in offering greater accuracy and specificity in assessing direct endothelial damage, in this CECinVOD multicentric study, we aimed to prospectively evaluate the potential role of CEC level in predicting and diagnosing SOS/VOD.

METHODS

Patients

Between October 2020 and November 2022, 170 patients across 11 Italian Bone Marrow Transplantation Units underwent prescreening in the CECinVOD study. Among them, 150 patients met the inclusion criteria: age $\geq$18 years, diagnosed with a hematologic neoplasm, undergoing allo-HCT with myeloablative conditioning (MAC), with a good performance status (Eastern Cooperative Cancer Group Performance Status 0-1 or Karnofsky Performance Status $\geq$90), normal serum creatinine value, and normal or $<$ 2.5 normal transaminase, alkaline phosphatase, and ɣGT values. In addition, these patients were able to provide blood samples taken at all the scheduled time points. The local Research Ethics Committee approved the study protocol, and all patients provided written informed consent, in accordance with the Declaration of Helsinki.

Study Design

The primary objective of the study was to evaluate the potential role of CECs as a biomarker for VOD risk and diagnosis in patients undergoing allo-HCT with MAC. The secondary endpoint was to assess the correlation between CEC level and the response to defibrotide treatment.

Serial blood sampling was performed in every patient at the following time points: before conditioning (T0), at the end of conditioning and before allo-HCT (T1), at the time of neutrophil engraftment (T2), and 7 to 10 days after engraftment (T3). In patients who developed VOD/SOS, additional blood samples were collected at any time of suspected or proven VOD onset (T4) and then weekly during defibrotide treatment (T5 to T8).

At each time point, the EASIX score was evaluated concurrently, as indirect marker of endothelial activation/injury in SOS/VOD [22]. The EASIX score was calculated using the following formula: lactate dehydrogenase (LDH) (U/L) $\times$ creatinine (mg/dL)/thrombocytes ($\times$ 10^9/L).

SOS/VOD Definition and Grading

SOS/VOD was defined according to the 2016 EBMT criteria [28]. "Classical" SOS/VOD was defined as occurring within 21 days after allo-HCT and presenting with bilirubin $\geq$2 mg/dL and any 2 of the following criteria: painful hepatomegaly, weight gain $>$5%, and ascites. "Late-onset" SOS/VOD was defined when classical VOD/SOS occurred beyond day 21 after allo-HCT or in presence of a histologically proven SOS/VOD beyond day 21 after allo-HCT, or if 2 of the following criteria were presented beyond day 21 after allo-HCT: bilirubin $\geq$2 mg/dL (or 34 μmol/L), painful hepatomegaly, weight gain $>$5%, ascites, and hemodynamic or/and ultrasound evidence of SOS/VOD.

SOS/VOD severity was also grading according to the 2016 EBMT criteria [28], based on the level of bilirubin and its rate of change, liver function (transaminase), weight increase, renal function, and the kinetics of their onset. This grading system is divided into 5 categories, as in the Common Terminology Criteria for Adverse Events: grade 1 = mild; grade 2 = moderate; grade 3 = severe; grade 4 = very severe; and grade 5 = death.

Patients with SOS/VOD were treated with defibrotide (25 mg/kg/day i.v. in 4 divided doses for a minimum of 3 weeks. If symptoms associated with SOS/VOD persisted, the treatment regimen was continued until resolution of VOD/SOS

indicators, for a maximum duration of 60 days [29]). Defibrotide is the only Food and Drug Administration/European Medicines Agency-approved drug for this disease. Complete response (CR) was defined as evidence of improvement in VOD/SOS-related symptoms concomitant with a reduction in bilirubin level to <2 mg/dL [30].

CEC Identification and Count

For CEC analysis, peripheral blood (PB) was collected in dedicated 10-mL tubes containing a cell preservative (CellSave Preservative Tubes; Menarini Silicon Biosystems). All samples were stored at room temperature, shipped via overnight express courier to the referral laboratory (DME-CTC Laboratory at Veneto Institute of Oncology [IOV]-Padua), and processed within 96 hours as described previously [31,32]. CECs were detected through the FDA-approved CellSearch system using the CellSearch Circulating Endothelial Cell Kit (Menarini Silicon Biosystems), according to the manufacturer's instructions. In brief, CECs were enriched from 4 mL of peripheral blood via anti-CD146-coated ferrofluid nanoparticles (a marker for endothelial cell lineage) and stained withs of fluorescent-labeled antibodies (anti-CD105-PE [endoglin protein expressed by endothelial cells], anti-CD45-APC [expressed by leukocytes] and DAPI [nuclear stain]). An event was classified as a CEC when its morphologic features were consistent with those of a cell and exhibited the phenotype CD146+, CD105+, DAPI+ and CD45- [33].

The CellSearch system is a semiautomated system that allows standardization of CEC identification and high levels of reproducibility, specificity, and sensitivity [26,31,32]. Counts were expressed as number of CECs per 4 mL of peripheral blood.

Statistical Analysis

Standard descriptive statistics were used to summarize the patient samples. Continuous data were expressed as median (range). Group comparisons were done using the Wilcoxon-Mann-Whitney rank-sum test or *t* test, and associations between categorical variables (2-way tables) were assessed using the Fisher exact test or chi-square test, as appropriate. The clinical and laboratory parameters were analyzed as possible factors related to CEC level. The cumulative incidence of SOS/VOD was calculated from the date of allo-HCT to the date of SOS/VOD occurrence, treating death without SOS/VOD as a competing event, according to the Fine and Gray test. Risk factors for VOD occurrence, severity, and outcomes were identified using univariate and multivariable Fine-Gray proportional hazards regression for competing risks. Multivariable analysis was conducted on variables with $P < .1$ in univariate analysis. To avoid multicollinearity issues, highly correlated predictors were identified using the Pearson correlation test and excluded from the multivariable analysis when associated with other factors in univariate analysis. All reported *P* values were 2-sided, and $P < .05$ was considered to indicate statistical significance.

Given the challenge in estimating the SOS/VOD incidence, our sample size was based on an expected incidence of 14% to 20% derived from published data. Therefore, for this explorative study, a sample size of 150 cases was considered adequate to diagnose VOD in at least 21 patients, aligning with the recommendations of Lancaster et al. [34].

Statistical analyses were performed with EZR software version 1.40 [35]. For original data, please contact mirko.farina@unibs.it.

RESULTS

Patients

A total of 150 patients were evaluated, including 98 females (65%), with a median age of 49.5 years (range, 18 to 69 years) (Table 1). Allo-HCT was performed with an HLA-matched familial donor in 27 patients (18%), an unrelated donor in 98 patients (65.3%), and a haploidentical donor in 25 patients (16.7%). Hematologic diseases included 84 cases (56%) of acute myeloid leukemia (AML), 28 cases (18.6%) of acute lymphoblastic leukemia, 18 cases (12%) of myelodysplastic syndrome, 18 cases (12%) of myeloproliferative neoplasm, 1 case (.7%) of multiple myeloma, and 1 case (.7%) of non-Hodgkin lymphoma. Sixty-five patients (43.4%) were in complete response (CR), 56 (37.3%) were in partial response/CR>2, and 29 (19.3%) were in progressive/stable disease at the time of transplant.

All patients received ursodeoxycholic acid as SOS/VOD prophylaxis, and no patient received defibrotide.

SOS/VOD

Among the 150 patients included in the study, 6 (4%) developed SOS/VOD during follow-up (Table 2). No cases of anicteric SOS/VOD were diagnosed. Three cases were classified as classical SOS/VOD, and the other 3 cases were categorized as late-onset according to the EBMT criteria [28]. The median time to onset of SOS/VOD after allo-HCT was 18.5 days. Among the affected patients, 4 experienced severe grade SOS/VOD, and 2 had a very severe presentation. Common clinical

Table 1
Patient Characteristics (N = 150)

Characteristic	Value
Age at transplantation, yr, median (range)	49.5 (18-69)
Male sex, n (%)	52 (35.0)
Disease, n (%)	
AML	84 (56.0)
MPN	18 (12.0)
ALL	28 (18.6)
MDS	18 (12.0)
NHL/MM	2 (1.4)
Disease status, n (%)	
CR	65 (43.4)
≥2 CR	42 (28.0)
PR	14 (9.3)
SD/PD	29 (19.3)
Lines of previous therapy, n (%)	
0-1	79 (52.7)
≥2	71 (47.3)
Cardiovascular risk factor, n (%)	16 (10.7)
Previous cardiovascular complications, n (%)	17 (11.3)
Donor type, n (%)	
HLA-matched related donor	27 (18.0)
HLA-matched unrelated donor	98 (65.3)
Haploidentical donor	25 (16.7)
Stem cell source, n (%)	
Peripheral blood	143 (95.3)
Bone marrow	3 (2.0)
Cord blood	4 (2.7)
CMV serology IgG, donor/recipient, n (%)	
IgG+/-	14 (9.3)
IgG-/+	39 (26.0)
Sorror HCT-CI, n (%)	
0-1	80 (53.0)
2	37 (25.0)
≥3	33 (22.0)
Conditioning regimen, n (%)	
High-dose busulfan	73 (49.0)
High-dose treosulfan	42 (28.0)
High-dose TBI-based regimen	32 (21.0)
GVHD prophylaxis, n (%)	
ATG	66 (53.0)
Sirolimus	11 (9.0)
SOS/VOD development (EBMT criteria), n (%)	6 (4.0)

MPN indicates myeloproliferative neoplasia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; CR, complete remission; SD, stable disease; PD, progressive disease.

manifestations included fluid retention with associated weight gain (n = 6), painful hepatomegaly (n = 4), and elevated bilirubin level (n = 4). All patients exhibited ultrasound (US) evidence of SOS/VOD, including 5 with hepatomegaly and ascites and 4 with portal vein dilatation. Elastography, performed in 3 of the 6 patients at the time of SOS/VOD onset, revealed higher liver stiffness measurements in 2 patients (77 kPa and 64 kPa).

All 6 patients were treated with defibrotide, the sole agent approved for this condition [36], and 5 patients (83%) achieved a CR. Conversely, 1 patient died from complications of VOD (mortality rate, 17%). Patients receiving a total body irradiation-based regimen showed a trend toward developing VOD compared to those receiving treosulfan (10 to 14 g/m^2) or busulfan (9.6 to 12.8 mg/kg) ($P = .08$; Table 2). We also evaluated whether the EASIX score had an impact on SOS/VOD onset in our cohort. A trend toward an association between SOS/VOD development and EASIX score was noted at the time of enrollment (T0) and after the start of conditioning (T1), but this did not reach statistical significance ($P = .19$ and .64, respectively). Conversely, at engraftment (T2), EASIX score was related to the development of late-onset SOS/VOD ($P = .002$; Tables 2 and 5). This result was confirmed by multivariate analysis (EASIX at T2: hazard ratio [HR], 1.07; 95% confidence interval [CI], 1.03 to 1.11; $P = .001$; Table 5).

CEC Count

A total of 615 samples were collected and analyzed, obtained at the standard time points (n = 4 for each patient) and at the additional time points for those patients who developed SOS/VOD. Considering the time points from T0 to T3, 584 out of the expected 600 samples were successfully collected and analyzed (successful sampling rate, 97.3%). Of the 16 missing samples, 6 were missing due to technical problems, and 10 samples were not sent for analysis by the different centers for logistical reasons. Samples at the additional time points (T4 to T8) were successfully collected and analyzed in 4 of the 6 patients who developed SOS/VOD.

At the time of enrollment (T0), AML patients had lower CEC levels ($P = .02$; Figure 1 and Table 3). No other clinical or laboratory characteristics were related to CEC level at T0 (Table 3).

Patients had higher CEC levels at the start of the conditioning regimen (T1: median, 12 CECs/mL; range, .75 to 309.75) than at baseline (T0; median, 9.13 CECs/mL; range, .25 to 924.0) (P = .02; Figure 2). In addition, CEC levels were significantly

Table 2
Clinical Characteristics According to VOD Onset

Characteristic	Patients with VOD (N = 6; 4)	Patients without VOD (N = 144; 96%)	*P* Value
Age, yr, median (range)	52.7 (28-68)	48.2 (18-69)	.39
Male sex, n (%)	1 (17)	51 (35)	.67
CEC level, median (range)			
T0	17.25 (4-39.25)	9 (.25-924.5)	.23
T1	20.6 (7.25-78.75)	12 (.75-42)	.26
T2	37.75 (12-72.25)	24.5 (1-120.25)	.58
T3	37.25 (13-66)	27.25 (1.8-295)	.35
CEC level, relative increase, %, median (range)			
T1 vs T0	104.8 (-81 to 343)	35.6 (-100 to 48,000)	.61
T2 vs T1	7.2 (-100 to 660)	111.3 (-100 to 8800)	.15
T4 vs T2 vs T3 vs T2	171 (69 to 283)	2 (-97 to 4560)	**.04**
T5 vs T4	93 (4 to 221)	NA	
T6 vs T5	-51 (-71 to 33)	NA	
T7 vs T6	-71 (-49 to -99)	NA	
Disease, n (%)			
AML	1 (17)	84 (58)	.09
MPN	2 (33)	16 (11)	.15
ALL	2 (33)	25 (17)	.29
MDS	0 (0)	18 (12,5)	>.99
NHL/MM	1 (17)	1 (1)	.08
Disease status, n (%)			
CR	2 (33.3)	63 (43.8)	.70
≥2 CR	2 (33.3)	40 (27.8)	.67
PR	2 (33.3)	12 (8.3)	.10
SD/PD	0 (0)	29 (20.1)	.60
Previous lines of therapy, n (%)			
0-1	2 (33.3)	77 (53.5)	.69
≥2	4 (66.7)	67 (46.5)	.69
Previous cardiovascular complications, n (%)	1 (17)	15 (10)	.50
Cardiovascular risk factor, n (%)	1 (17)	16 (11)	.52
Donor type, n (%)			
Unrelated donor	2 (33)	93 (65)	.19
HLA-mismatched donor	4 (67)	49 (34)	.19
Stem cell source, n (%)			
Peripheral blood	6 (100)	137 (95)	>.99
Bone marrow	0 (0)	3 (2)	>.99
Cord blood	0 (0)	4 (3)	>.99
CMV serology IgG D/R (N = 134), n (%)			
IgG+/+	1 (17)	69 (48)	.19
IgG+/-	0 (0)	14 (10)	>.99
IgG-/-	2 (33)	10 (7)	.06
IgG-/+	2 (33)	37 (26)	.63
Sorror HCT-CI, n (%)			
0-1	2 (33)	76 (53)	.42
2	1 (17)	35 (24)	>.99
≥3	3 (50)	29 (20)	.12

(*continued*)

Table 2 (*Continued*)

Characteristic	Patients with VOD (N = 6; 4)	Patients without VOD (N = 144; 96%)	*P* Value
Karnofsky Performance status, n (%)			
90-100	6 (100)	140 (97)	>.99
0-80	0 (0)	4 (3)	>.99
Conditioning regimen, n (%)			
High-dose busulfan	2 (33)	71 (49)	.68
High-dose treosulfan	1 (17)	41 (28)	.67
High-dose TBI-based	3 (50)	29 (20)	.08
GVHD prophylaxis, n (%)			
Sirolimus	1 (17)	10 (7)	.27
Serum bilirubin >1.5 mg/L (>26 μmol/L) at T0, n (%)	3 (50)	25 (17)	.08
Transaminase >2.5 ULN at T0, n (%)	0 (0)	9 (6)	>.99
EASIX, median (range)			
T0	2.56 (.86-4.45)	1.39 (.17-111.47)	.19
T1	3.73 (.88-10.49)	2.54 (.05-106.75)	.64
T2	24.97 (13.25-47.30)	4.31 (.01-47.24)	**.002**

Categorical variables are summarized as number and percentage and compared using the unpaired *t* test or chi-square test, as appropriate. Continuous variables are summarized as median and range and compared using the Mann-Whitney *U* test. $P \leq .05$ was considered to indicate statistical significance. CECs are expressed as number/mL. Bold type indicates statistical significance.

higher at the time of engraftment after allo-HCT (T2: median, 24.5 CECs/mL; range, 1.0 to 298.75) than at the time of conditioning (T1) ($P < .0001$; Figure 2). No differences were seen between CEC levels at 1 week after engraftment (T3: median, 27.4 CECs/mL; range, 1.75 to 295.0) and at the time of engraftment (T2) ($P = .91$).

Patients who developed SOS/VOD had higher median levels of CECs at all analyzed time points, although the differences did not reach statistical significance (Figure 3). Specifically, the median CEC count at T0 (preconditioning) was 17.25 CECs/mL (range, 4 to 39.25) in patients who developed SOS/VOD, compared with 9.0 CECs/mL (range, .25 to 924.0) in those who did not develop SOS/VOD (P = .23; Figure 3). Before allo-HCT (T1), the median CEC count was 20.63 CECs/mL (range, 7.25 to 78.75) in patients who developed SOS/VOD and 12.0 CECs/mL (range, .75 to 309.0) in those who did not ($P = .26$).

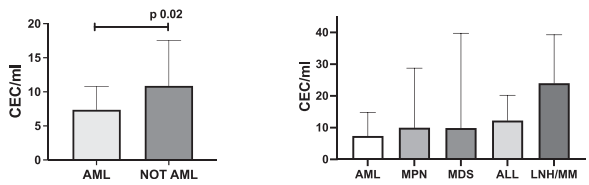

Figure 1. CEC/mL before conditioning regimen (T0) according to the type of disease. AML patients had lower CEC levels compared to patients diagnosed with other diseases. The analysis was performed using the Mann-Whitney test. *Statistical significance with $P < .05$. Error bars represent standard deviation.

Patients with more a CEC count >17 CECs/mL at baseline had a higher risk of developing VOD/SOS (HR, 5.09; 95% CI, .95 to 27.43; $P = .05$). Indeed, these patients had a higher cumulative incidence of VOD/SOS at day 21 (4.7% [95% CI, .8% to 14.0% versus .9% [95% CI, .1% to 4.6%]), as well as at day 100 (9.3% [95% CI, 2.9% to 20.3%] versus 1.9% [95% CI, .4% to 6.0%]; $P = .04$; Figure 4A). The threshold of 17 CECs/mL was identified by a receiver operating characteristic curve analysis, with an area under the curve of .648 (95% CI, .459 to .838). Additionally, a bilirubin level >1.5 times the normal value and having a haploidentical donor were associated with a higher risk of VOD/SOS (cumulative incidence at day 100, 3.6% versus 1.7% [$P = .048$] and 14% versus 2.7% [$P = .02$], respectively; Figure 4). However, in multivariate analysis, only having a haploidentical donor was significantly associated with a higher risk of developing VOD/SOS (HR, 6.7, 95% CI, 1.2 to 36.8; $P = .03$; Table 4).

At SOS/VOD onset (T4), patients consistently exhibited higher CEC levels compared to the previous time point (T2 or T3), with increases ranging from +10% to +283% (median, +115%). Moreover, in patients who developed SOS/VOD after engraftment (T2), the relative increase in CEC count (at T4 from T2) was +171% (range, +69% to +283%) in patients with SOS/VOD versus +2% (range, -97% to

Table 3
CEC Levels at T0 by Patient Clinical Characteristics

Characteristic	CEC Level at T0, no./mL, median (range)	*P* Value
Sex		.96
Male	8.5 (1-924.5)	
Female	9 (.25-346.5)	
Disease		
AML	7.38 (.5-924.5)	**.02**
MPN	10 (.25-225)	.84
ALL	11.75 (2.75-128.5)	.15
MDS	9.88 (2-387)	.20
NHL/MM	24 (8.75-39.25)	.43
Disease status		
CR	10.63 (1-924.5)	.59
≥2 CR	10.25 (1.25-346.5)	.39
PR	9.75 (.25-40.5)	.98
SD/PD	7.75 (.5-225)	.77
Previous lines of therapy		
0-1	10.5 (1-924.5)	.50
≥2	8.75 (.25-346.5)	
Previous cardiovascular complications (N = 145)	11.25 (1.5-39.25)	.74
Cardiovascular risk factor	8 (.5-225)	.37
CMV serology IgG^+ R (N = 136)	9 (.25-924.5)	.64
Sorror HCT-CI (N = 140)		
0-1	11 (.5-387)	.69
2	7.25 (0.25-924.5)	.14
≥3	10.5 (2-103)	.27
Karnofsky Performance Status (N = 147)		
90-100	9 (.25-924.5)	.42
0-80	12 (10.5-20.25)	
Serum bilirubin >1.5 mg/L (>26 μmol/L) at T0 (N = 147)	11.75 (1.75-225)	.15
Transaminase >2.5 ULN at T0	10.75 (1-103)	.78

Bold type indicates statistical significance.

4560%) in patients who did not develop SOS/VOD (at T3 to T2; $P = .04$; Table 2). A relative increase in CEC count >1.5-fold was confirmed as a predictive factor associated with the development of late-onset VOD/SOS in multivariate analysis (T4-T2 versus T3-T2 >150%: HR, 10.54; 95% CI, 1.34 to 83.21; $P = .025$; Table 5), along with a high EASIX score at engraftment (Table 5).

After the start of defibrotide treatment (T4), CEC level showed an initial increase in the first week (T5), followed by progressive decreases during VOD treatment (T6 and T7; median reduction, -50,7% and -71,5%, respectively; Figure 5A). In contrast, transaminase, LDH, and creatinine levels decreased rapidly after the start of defibrotide therapy, whereas platelet count and bilirubin did not improve in all patients analyzed after the initiation of defibrotide (Figure 5B).

In some cases, CECs have been observed as clusters of multiple cells at the different time points. However, the presence of CEC clusters at baseline was not associated with the development of VOD/SOS. One out of 6 patients who developed VOD had CEC clusters (20%), compared to 26 out of 118 patients who did not develop VOD (22%) ($P > .99$). Similarly, the presence of CEC clusters at other time points was not correlated with VOD development, including at VOD onset or during defibrotide treatment. Nevertheless, the presence of CEC clusters was significantly associated with the CEC count (Figure 6), whereas no other relationship between clinical and laboratory characteristics was related to the presence CEC clusters.

Because the EASIX score is considered a marker of endothelial activation/dysfunction, we investigated whether CEC level may be related to it. However, we did not find any relationship between EASIX and CEC level at any time point (Table 3).

DISCUSSION

Over the last several decades, various efforts have been undertaken to facilitate early diagnosis of SOS/VOD, but to date, studies have failed to identify a biomarker that can reliably predict this complication or aid its diagnosis [2]. With the CECinVOD study, we prospectively evaluated the levels of CECs in patients undergoing MAC allo-HCT to assess their potential role in predicting and diagnosing SOS/VOD. This marks the first evaluation of CECs in relation to SOS/VOD development, enabling a direct assessment of endothelial injury. Conversely, previous studies focused on the role of indirect endothelial biomarkers [16] and failed to establish a clear relationship with SOS/VOD [17,37,38].

Six of our 150 patients developed SOS/VOD, for an incidence rate of 4%. This incidence is low compared with previous reports [6,39-42], in which MAC regimens have been associated with an elevated risk of SOS/VOD [1,43]. However, it is consistent with the recent EBMT survey [1] showing a 2.4% cumulative incidence of SOS/VOD at 100 days. Given that most published data are derived from retrospective studies [6,44] characterized by high heterogeneity in study

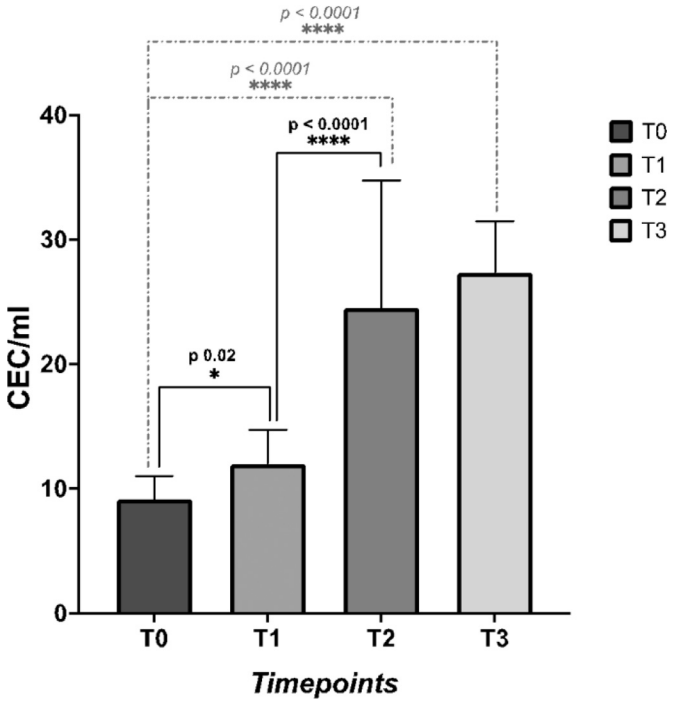

Figure 2. CEC/mL of peripheral blood at different time points. T0, prior to conditioning; T1, after conditioning; T2, at engraftment; T3, 1 week after engraftment. The analysis was performed using the Wilcoxon test. *Statistical significance with $P < .05$. ****Statistical significance with $P < .0001$.

populations and use of more toxic transplantation procedures, cross-study incidence comparisons are challenging. However, our observation confirm an apparently progressive decrease in SOS/VOD incidence in recent years, as proposed by other authors [8,45].

Patients with AML had lower CEC levels at baseline (Figure 1), possibly due to the lower degree of endothelial involvement in AML compared to other hematologic diseases, such as myelofibrosis [32] and others myeloproliferative neoplasms [25]. CEC count was elevated after

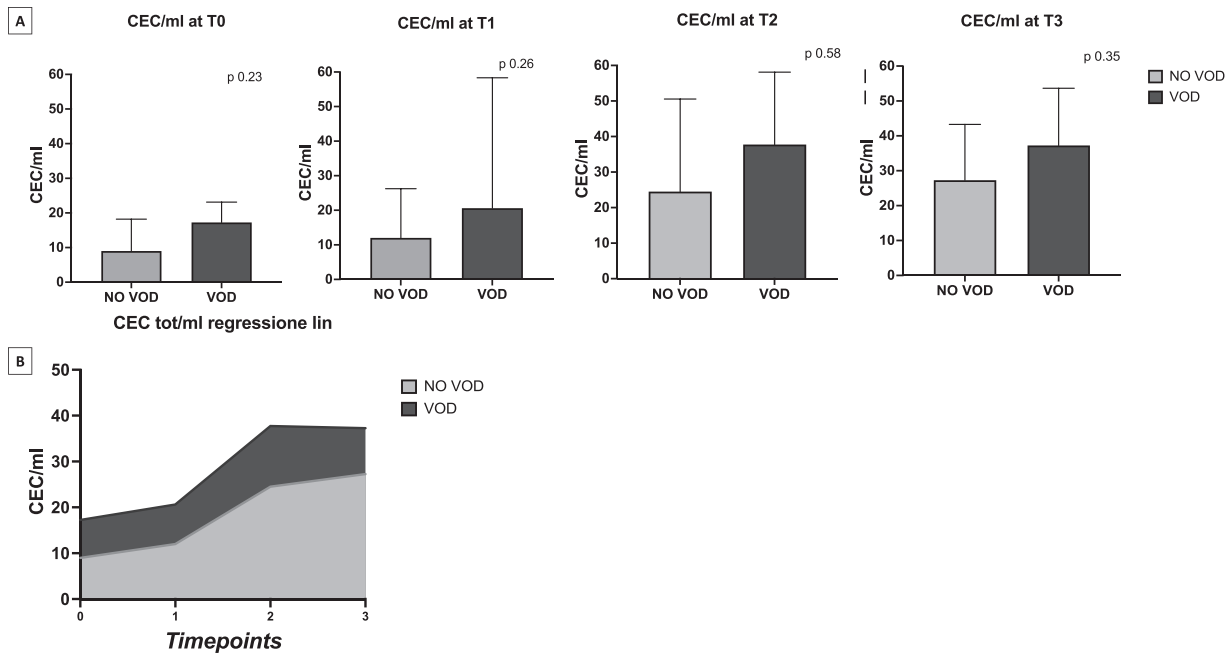

Figure 3. CEC levels at different time points in patients with SOS/VOD (black) and those without SOS/VOD (gray).

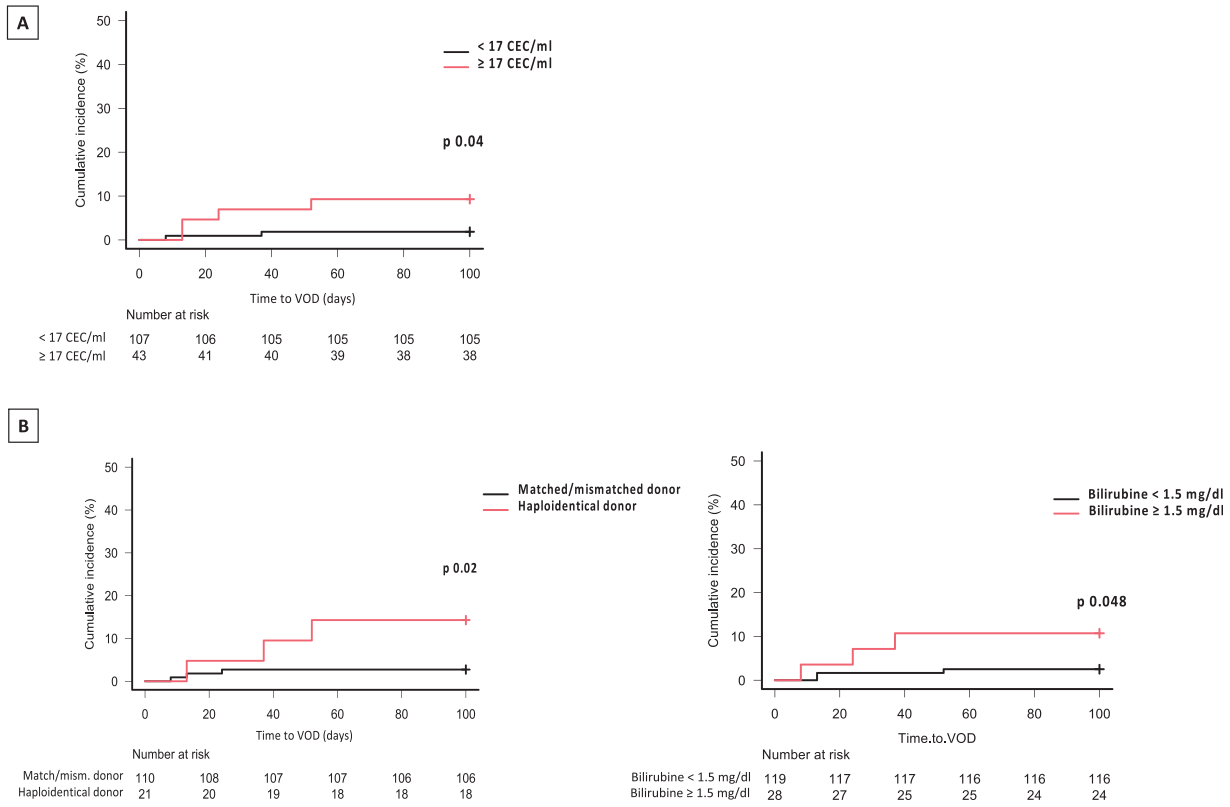

Figure 4. (A) Cumulative incidence of SOS/VOD assessed based on baseline CEC level (T0), categorized as >17 CECs/mL (red) or <17 CECs/mL (black). Patients with >17 CECs/mL at T0 exhibited a higher incidence of VOD/SOS. Specifically, the cumulative incidence at day 21 was 4.7% (95% CI, .8% to 14.0%), compared to 1.9% (95% CI, .4% to 6.0%) in patients with <17 CECs/mL. At day 100, the cumulative incidence in the 2 groups rose to 9.3% (95% CI, 2.9% to 20.3%) and 1.9% (95% CI, .4% to 6.0%), respectively ($P = .04$). (B) Bilirubin level >1.5 mg/dL (red) at baseline and having a haploidentical donor (red) were associated with a higher cumulative incidence of SOS/VOD. The analysis was conducted using the Fine-Gray test.

conditioning ($P = .02$), and they further increased at engraftment ($P < .0001$), underscoring the endothelial damage in patients undergoing allo-HCT, as described previously [26,27]. Interestingly, patients with higher CEC levels were more likely to have CEC clusters (Figure 6), possibly due to cell activation and an increased potential for cell aggregation, possibly favored by other soluble factors. This is the first description of CEC clusters in this context, and further investigation is needed to clarify their biological and clinical significance.

Table 4
Multivariate Analysis of Factors Predictive of SOS/VOD Onset at the Time of Enrollment (T0)

Variable	HR	95% CI	*P* Value
Donor type, haploidentical vs others	6.72	1.23-36.81	**.03**
Serum bilirubin >1.5 mg/d	4.74	.76-29.66	.10
CECs, 17 cells/mL	3.73	.61-22.65	.15

The analysis used Fine-Gray proportional hazard regression to account for competing events. Bold type indicates statistical significance.

Baseline CEC counts >17 CECs/mL were associated with a higher incidence of SOS/VOD, along with bilirubin level >1.5 mg/mL and a haploidentical donor hematopoietic stem cell source. The latter 2 factors have been previously linked to VOD onset [1,2,46].

Patients who developed SOS/VOD consistently exhibited elevated CEC levels across all analyzed time points (Figure 3), although the differences did not reach statistical significance, likely due to the limited number of patients in the VOD group. A notable increase in CEC levels was observed at SOS/VOD onset, particularly in late-onset cases ($P = .04$). This finding was confirmed by multivariate analysis (CEC relative increase >150%), alongside the EASIX score at engraftment. Thus, CEC kinetics may be helpful when the diagnosis of SOS/VOD is more challenging.

Combining a CEC level ≥17/mL with other clinical or laboratory characteristics, such as use of a

Table 5
Univariate and Multivariate Analysis to Evaluate CEC Values as Independent Predictor Variable for Late-Onset SOS/VOD Development at the Time of Hematopoietic Engraftment

Variable	HR	95% CI	*P* Value
Univariate analysis			
CEC relative increase (T4-T2 vs T3-T2) > 150%	12.9	1.2-138.2	**.03**
Bilirubin >1.5 mg/L at T2	7.3	.69-78.3	.09
EASIX at T2	1.1	1.02-1.15	**.05**
TBI-based regimen	3.8	.7-18.2	.10
Diagnosis of AML vs others	.15	.01-1.236	.08
Multivariate analysis			
Diagnosis of AML vs others	.61	.04-7.90	.700
Bilirubin >1.5 mg/L at T2	6.67	.71-62.34	.096
EASIX at T2	1.07	1.03-1.11	**.001**
CEC relative increase (T4-T2 vs T3-T2) > 150%	10.54	1.34-83.21	**.025**

The analysis used Fine-Gray proportional hazards regression to account for competing events. Bold type indicates statistical significance.

haploidentical donor or bilirubin >1.5 mg/L and/or a relative increase in CEC level and higher EASIX score at engraftment could be useful for developing a risk score for SOS/VOD and aid the diagnostic process.

CEC kinetics also correlated with defibrotide treatment. Following an initial increase, CECs returned to pre-SOS/VOD onset levels after 3 weeks of therapy. This finding contrasted with the kinetics of other indirect markers of VOD-related tissue damage, which rapidly improved after treatment initiation. Clinically, these observations support the recommendation that treatment be continued for at least 3 weeks to effectively restore endothelial damage.

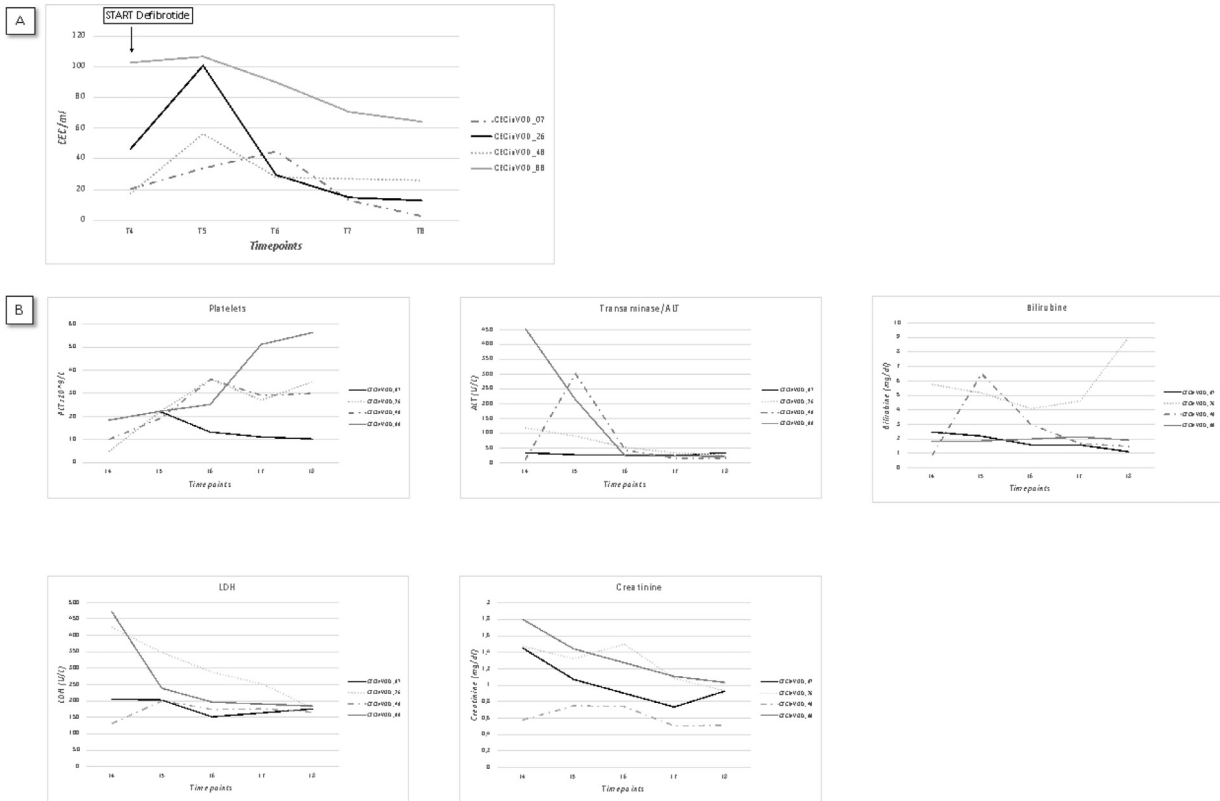

Figure 5. CEC levels (A) and platelet, transaminase, bilirubin, LDH, and creatinine kinetics (B) after the start of defibrotide treatment for SOS/VOD.

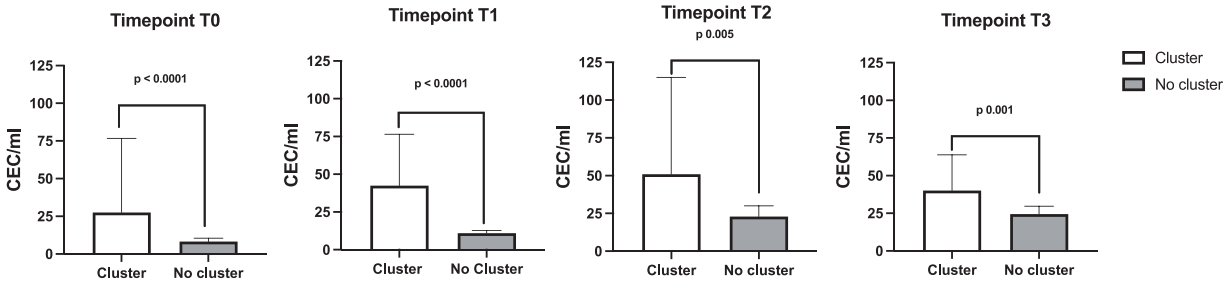

Figure 6. CEC clusters related to CEC levels at each time point.

CONCLUSION

The results of this multicenter prospective study suggest that CEC count measured by the FDA-approved CellSearch system is a valuable tool for directly evaluating the risk for and diagnosing SOS/VOD in patients undergoing allo-HSCT. High CEC levels at baseline and CEC kinetics, either alone or in combination with other factors, such as haploidentical donor, bilirubin level, and EASIX, can identify patients at increased risk of developing SOS/VOD and aid the diagnosis of this complication, especially in late-onset cases. CEC kinetics also can serve as a valuable tool to provide information on the optimal timing for discontinuation of defibrotide therapy.

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